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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,,251,265,** on October 21, 1998, by **UNIVERSITE DE SHERBROOKE.,** assignee of Adrien Beaudoin and Geneviève Martin, for "Process for Lipid Extraction of Aquatic Animal Tissues Producing a Dehydrated Residue".

PRIORITY DOCUMENT

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S. Gregoire

Agent certificateur/Certifying Officer

November 16, 1999

Date

Canada

(CIPO 68)

OPIC CIPO

ABSTRACT OF THE DISCLOSURE

The procedure includes the suspension of freshly collected material in an equal volume of acetone under inert gas atmosphere. Lipids are extracted by successive acetone and ethanol treatments. The procedure produces two lipid fractions and a dry residue enriched in protein and other material insoluble in organic solvents. Recovery of total lipids is comparable or superior to the Folch et al. (1957) procedure. It has been tested with krill, *Calanus* and fish tissues.

TITLE OF THE INVENTION

PROCESS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES PRODUCING A DEHYDRATED RESIDUE

FIELD OF THE INVENTION

The present invention relates to a method for lipid extraction of animal tissues and to the lipid and dry residue fractions obtained therefrom. More particularly, the present invention relates to a lipid extraction method using krill, *Calanus* and fish tissues as starting material.

SUMMARY OF THE INVENTION

Extraction process

Fresh (or frozen) material (*Euphausia pacifica* and other species) is suspended in cold acetone for a given period of time at low temperature (5 C or low r). A ratio of krill-acetone 1:6 (w/v) and an incubation time of 2 h in acetone were found to be optimal. Alternatively the material can be kept in an equal volume of acetone at low temperature for long periods of time (months) under inert atmosphere. The size of the material is an important factor for the penetration of acetone. Indeed, it is preferable to grind material with dimensions superior to 5 mm before getting it in contact with acetone. The suspension is swirled for a short period of time (about 20 min) after acetone addition. After filtration on an organic solvent resistant filter (metal, glass or paper) the residue is washed with two volumes of pure acetone. The combined filtrates are evaporated under reduced pressure. The water residue obtained after evaporation is allowed to separate from the oil phase (fraction I) at low temperature. The solid residue collected on the filter is suspended and extracted with two volumes (original volume of frozen material) of 100% ethanol. The ethanol filtrate is evaporated leaving a second fraction of lipids (identified as fraction II).

Variations of the process

Variable volumes of acetone relative to the levels of sample can be used. It is also applicable to the volume of acetone used to wash and to the volume of ethanol used to extract. Incubation times in solvents may vary. Particle size affect the recovery of lipids and the material could be ground in various sizes of particles, depending on the grinder used. Temperature of the organic solvents and temperature of the sample are not critical parameters, but it is preferable to be as cold as possible.

Methods

To compare the efficiency of the extraction process, a classical technique (Folch et al. 1957) implying chloroform and methanol was applied to krill. This is the standard of reference for the efficiency of the extraction process. Lipid recovery was estimated by suspending lipid fractions in small volumes of their original solvents and measuring by gravimetry small aliquots after evaporation.

To analyze lipid composition, small aliquots of the various extracts were loaded on silica-gel plates and fractionated by thin layer chromatography, TLC (Bowyer et al. 1962) with the following solvents. Neutral lipids: hexane, ethyl ether, acetic acid (90:10:1 v/v) and phospholipids: chloroform, methanol, water (80:25:2 v/v). Fatty acid composition of *E. pacifica* was analyzed by gas liquid chromatography, GLC (Bowyer et al. 1962) including some modifications to the original technique: 1h at 65°C instead of 2h at 80°C, three washes with hexane instead of two and no wash with water.

The dry residue is wetted with ethanol to facilitate a progressive rehydratation of the proteins.

To get rid of traces of organic solvents, lipid fraction I and II are warmed (60°C for fraction I and 70°C for fraction II) for 5 min under inert atmosphere.

Applications

The different fractions (oil, proteins, and others) of aquatic animal biomass xtracted by the current procedure could be used in many fields:

1-Aquaculture

As mentioned in results, fatty acids 20:5 (eicosapentaenoic acid) and 22:6 (docosahexaenoic acid) are found in high concentrations in krill, *Calanus*, and fish. Farming fish on high quality marine oils rich in docosahexaenoic and eicosapentaenoic (EPA) acids is an efficient means of delivering these essential nutrients in human diets and also efficiently exploiting a strictly limited marine bioresource (Sargent 1997). Krill may be used as food supplement for fish and shrimp (Sargent 1997) because of its capacity to improve growth and survival capacity against diseases (Runge 1994), as pigmentation enhancer for ornamental fish species and as starter diet for marine and fresh water species (Prawn Hatchery Food 1997).

2-Nutraceuticals

Considering the beneficial effects of omega-3 fatty acids, the marine oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. 22:6 *n*-3 fatty acid is essential for proper development of the brain and the eye (Sargent 1997).The beneficial effects of *n*-3 polyunsaturated fatty acids in reducing the incidence of cardiovascular disease by lowering plasma triacylglycerol level and altering platelet function towards a more anti-atherogenic state has been reviewed (Christensen 1994). Also, dietary krill oil, like fish oil, can suppress the development of autoimmune murine lupus: EPA substitutes for arachidonic acid, a substrate for cycloxygenase thereby reducing the production of prostaglandins (Chandrasekar 1996). The effects of dietary supplementation with w-3 lipid-rich krill oil includes decreased expression of TGF_{β} in kidneys and of the oncogene–c-*ras* in splenocytes (Chandrasekar 1996). Krill oil has beneficial effects on life span and amelioration of renal disease similar to those previously described in studies with fish oil (Chandrasekar 1996).

3-Animal food

Feeding the animals with omega-3 fatty acids may increase the level of unsaturated fatty acids and decrease cholesterol levels of meat. This property is exploited in the poultry industry to improve the quality of eggs. *Calanus*, in particular, is a full of promise ingredient of domestic animal's food (Runge 1994).

4-Cosmetic industry

Calanus is used for the production of moisturizing creams (Runge 1994).

5-Medical applications

Krill may be used as a source of enzymes for medical application like the debridement of ulcers and wounds (Hellgren 1991) or to facilitate food digestion.

Finally, these marine products are also rich in liposoluble vitamins A, D, E and K and carotenoids that are extracted with lipids. The chitin of krill and *Calanus* could be exploited to protect plants against fungi. Also, marine oils contain unidentified antioxidants which may have potential therapeutic properties.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Results

Note on experimental conditions

The lipid extraction with acetone, then ethanol is practicable under different experimental conditions, as mentioned on page 1 of this document (variation of the process). Moreover, the majority of data shown in this document are from experiments made with sample-acetone ratio of 1:9 (w/v) incubated overnight at 4°C and with sample-ethanol ratio of 1:4 (w/v) incubated 1h at 4°C. In addition, no material has been ground in most experiments. Only later, tests have been made to standardize the method for extraction of lipids with acetone, then ethanol. As shown in Figure 9 and 11, it appears that optimal ratios of sample-solvent are 1:6 (w/v) for acetone and 1:2 (w/v) for ethanol. Figure 10 and Figure 12 show that optimal incubation times are 2 h for the first solvent and 30 min for the second. Grinding has been experimented and it is clear that solvents have a better impact on ground material, as shown in Table 5. Then, experimental conditions are specified for each experiment.

Diagram 1 illustrates the procedure of lipid extraction from frozen krill which is the same used with dry krill and other fresh species as *Calanus*, mackerel, trout and herring.

Interpretation of results

Table 1 shows that higher levels of lipids are extracted by acetone followed by ethanol as compared to the classical procedure of Folch et al. (1957). The same information is found in Table 5 concerning another krill species (*Megayctiphanes norvegica*). Back to Table 1, one can see that the combination of acetone and ethanol as a single step did not improve the extraction process.

Table 2 shows the results of lipid extraction from frozen *Euphausia pacifica*, a species of krill from Pacific Ocean. Assuming an eighty percent content of water, the lipid content is comparable to dry krill as shown in Table 1. Samples of *E. pacifica* incubated in different ratios of acetone at 4°C for 112 days have been inoculated on NA medium containing Bacto beef extract 0,3%, Bacto peptone 0,5% and Bacto agar 1,5% (Difco 1984) then incubated at room temperature or 4°C for 18 days. No significant bacterial growth was observed at a ratio of 1 volume of acetone per gram of krill. At higher proportions of acetone (2 volumes and 5 volumes), there was no bacterial growth at all, which means that acetone preserves krill samples. Acetone is known as an efficient bactericidal and viricidal agent (Goodman et al. 1980).

Table 3 shows the yield of lipids from *M. norvegica*. The percentage of lipids is lower (3,67 %) than for *E. pacifica* (4,04 %) shown in Table 2. These variations can be attributable to the season of catch.

Table 4 shows the krill composition obtained from experiments 3 and 4 with frozen *E. pacifica* (Table 2). One finds about 83% of water, 4% of lipids and 12% of dry residue.

Table 5 shows the influence of grinding on the efficiency of extraction of *M. norvegica* lipids. These extractions were carried out under optimal conditions and show the definite advantage of the procedure over the classical method (4,46 % versus 3,30 %). It also shows that grinding may be an important factor wh n the species is large (4,46% versus 3,53 %).

Considerable quantity of lipids were obtained from *Calanus* (Table 6). Some variations in *Calanus* species composition may explain the variations between experiments 1 and 2 (8,22 % and 10,90 % of fresh weight).

When the technique was applied to fish (mackerel) peripheral tissues (mainly muscles) or viscera, an amount of lipids was extracted (Table 7) but it appeared less efficient than the classical method since extractions of the residue with the latter technique allowed us to recover less lipid. Overall, our technique would allow us to exploit parts of fish that are usually wasted after the withdrawal of fillets of the fish or lipid extracts from fishes not used for human consumption. Those fish tissues not used after the transformation of the fish for human consumption could be stored in acetone, then lipids could be extracted with our process. Extraction of lipids from trout and herring were carried out in parallel with the classical method. Results appear in Table 8 and 9. The yield is not significantly different for the viscera whereas with peripheral tissues (muscles) the classical technique is superior (14,93 % versus 6,70 %). Technique using acetone followed by ethanol for trout and herring (and maybe for other species) seems applicable as well as for mackerel. Table 11 shows the suggested procedure for lipid extraction of aquatic animal tissues.

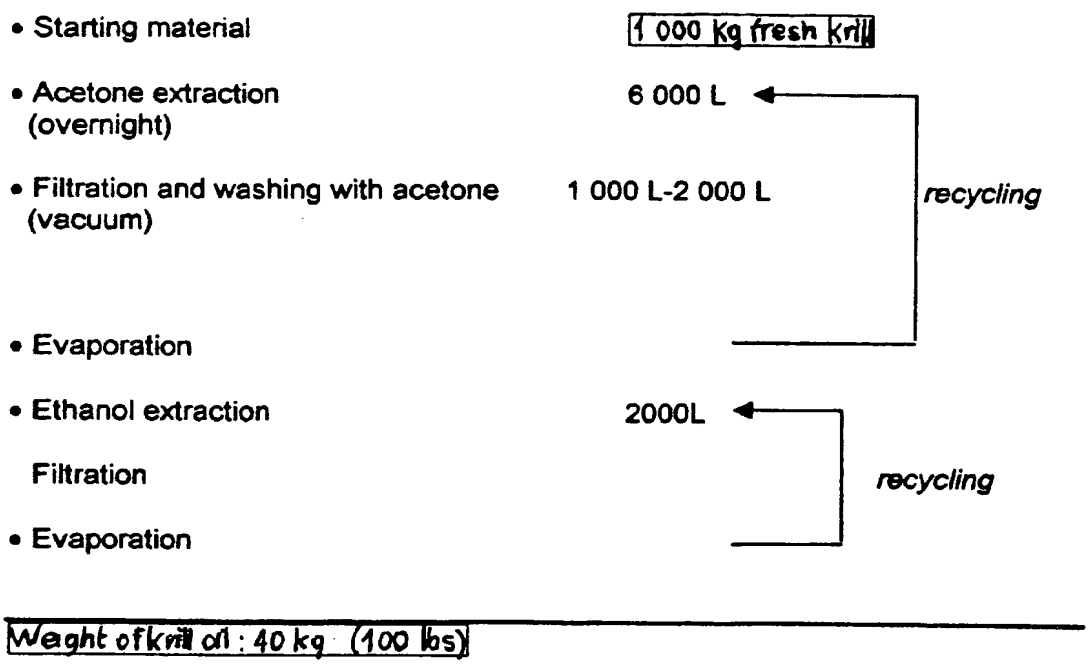
Figures 1 to 4 show chromatograms of fatty acid composition of *E. pacifica* lipids. On each of them, high proportions of 20:5 and 22:6 fatty acids (characteristic of marine oils) are noticeable and represented by two distinct peaks. The concentration of the sample on Figure 4 was lower than the others, so the peaks don't have the same amplitude. With retention times and amounts gave by the chromatograph, identification and compilation of the majority of the fatty acids have been done (see Table 10).

Figures 5 to 8 (TLC) show a higher proportion of neutral lipids as compared to phospholipids in marine oils.

The influence of incubation time on the efficiency of the acetone to extract lipids from *E. pacifica* is illustrated in Figure 9. Extraction is already completed at 2 h. With this time, we proceeded to determine the influence of the sample-acetone ratio (Figure 10). Results show that a ratio of 1:6 (w/v) produce the best yield. The second lipid extraction is carried out with ethanol. The incubation time in this solvent should be at least 30 min as indicated by the results of Figure 11. The volume of ethanol does not appear to be critical since the same yield was obtained with different volumes of ethanol.

One of the inventors, Mr Adrien Beaudoin, has tasted the different lipid fractions. No side effect was observed. The fraction I has the taste of the cod liver oil and the insoluble material tastes like salty shrimps.

DIAGRAM 1. KRILL LIPID EXTRACTION PROCESS



Weight of krill oil : 40 kg (100 lbs)

TABLE 1. EXTRACTION OF DRY KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	8,00	
	ethanol [b]	7,60	15,60
2-	"	19,70	
		6,90	26,60
3-	"	8,15	
		11,20	19,35
4-	"	6,80	
		13,60	20,40
			$\bar{x}$=20,49
			σ= 3,95
5-	Chlor : MeOH [c]		15,60
6-	"		14,90
			$\bar{x}$=15,20
			σ= 0,30
7-	Combined acetone-ethanol [d]		14,30

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), no incubation.

[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 night at 4°C.

[c] :Folch et al. 1957

[d] :Extraction made with a sample-acetone-ethanol ratio of 1:5:5, no incubation.

TABLE 2. EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	2,26	
	ethanol [b]	2,14	4,40
2-	"	2,25	
		1,13	3,38
3-	"	2,71	
		1,80	4,50 [c]
4-	"	2,94	
		1,45	4,39 [c]
5-	"	2,44	
		1,43	3,87
6-	"	2,54	
		1,23	3,77
7-	"	2,58	
		1,46	4,04
8-	"	2,48	
		1,39	3,87
9-	"	2,46	
		1,72	4,18
			$\bar{x}$=4,04
			σ=0,34

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.

[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1h at 4°C.

[c] :See Table 4 for total composition.

TABLE 3. EXTRACTION OF FROZEN KRILL LIPIDS (*M. norvegica*)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a)]	1,82	
	ethanol [b)]	1,82	3,64
2-	"	1,15	
		2,35	3,50
3-	"	1,68	
		2,19	3,87
			$\bar{x}$=3,67
			σ=0,15

Determinations in triplicates (variation < 5 %).

[a)] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.

[b)] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 h at 4°C.

TABLE 4. FROZEN KRILL COMPOSITION (*E. pacifica*) on a fresh weight basis

Exp. No.	Lipids	Insoluble material	Water
3-	4,50	12,50	83,00
4-	4,39	11,50	84,11
	$\bar{x}$=4,44 σ=0,05	$\bar{x}$=12,00 σ= 0,50	$\bar{x}$=83,55 σ= 0,55

Determinations in triplicates (variation < 5 %).
Experience numbers refer to Table 2.

TABLE 5. INFLUENCE OF GRINDING ON EXTRACTION OF FROZEN KRILL LIPIDS (*M. norvegica*)

Exp. No.	Technique	Krill ground before 1st extraction	Yield (%)	Total (%)
1-	acetone [a]	yes	3,10	
	ethanol [b]		1,07	4,17
2-	"	no	2,14	
			1,39	3,53
3-	"	yes	3,32	
			1,14	4,46
4-	Chlor : MeOH [c]	yes		3,30
5-	"	yes		3,26

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-acetone ratio of 1:6, incubated 2 h at 4°C
[b] :Extraction made with a sample-ethanol ratio of 1:2, incubated 30 min at 4°C.
[c] :Folch et al. 1957.

TABLE 6. EXTRACTION OF FROZEN *Calanus* LIPIDS (*Calanus* sp.)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	6,18	
	ethanol [b]	2,04	8,22
2-	"	8,64	
		2,26	10.90
			$\bar{x}$=9,56
			σ=1,34

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.
[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 h at 4°C.

TABLE 7. EXTRACTION OF FRESH FISH.LIPIDS (Mack rel)

Exp. No.	Technique	Yield (%)	Total (%)
1-viscera	acetone [a]	6,11	
fish 1	ethanol [b]	0,59	6,70
2-tissues	"	3,78	
fish 1		0,91	4,69
3-viscera	"	10,46	
fish 2		0,57	11,03
4-tissues	"	6,65	
fish 2		1,41	8,06
5-viscera	"	8,39	
fish 3		0,66	9,05
6-tissues	"	5,27	
fish 3		0,97	6,24
7-viscera	"	8,47	
fish 4		0,69	9,16
8-tissues	"	8,40	
fish 4		1,02	9,42
9-viscera	Chlor:MeOH [c]		0,52
fish 1			
10-tissues	"		1,45
fish 1			

[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubation time:
- -fish 1 viscera: 4h, fish 1 tissues: 23h
- -fish 2 viscera: 23h45, fish 2 tissues: 45h30
- -fish 3 viscera: 8 days 2h20, fish 3 tissues: 8 days 22h30
- -fish 4 viscera: 17 days 23h, fish 4 tissues: 18 days 2h25

[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1h at 4°C.
[c] :Folch et al. 1957.

TABLE 8. EXTRACTION OF FRESH FISH LIPIDS (Trout)

Exp. No.	Technique	Yield (%)	Total (%)
1-viscera	acetone [a)]	34,70	
	ethanol [b)]	2,18	36,88
2-tissues	"	5,53	
		1,17	6,70
3-viscera	Chlor:MeOH [c)]		39,81
4-tissues	"		14,93

Determinations in triplicates (variation < 5 %).

[a)] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.

[b)] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 h at 4°C.

[c)] :Folch et al. 1957.

TABLE 9. EXTRACTION OF FRESH FISH LIPIDS (H rring)

Exp. No.	Technique	Yield (%)	Total (%)
1-tissues and viscera	acetone [a]	2,09	
	ethanol [b]	0,68	2.77
2-tissues and viscera	Chlor:MeOH [c]		5.95

Determination in triplicates (variation < 5 %).
[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°.
[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 h at 4°C.
[c] :Folch et al. 1957.

Table 10: Fatty acid composition *E. pacifica*

Solvent	Saturated	Unsaturated				Unidentified	
		Mono	Di	Poly	H-Poly		
chlo-meth	26,18	22,54	1,91	4,31	26,34	18,72	
acetone	21,4	22,18	1,75	4,67	24,52	25,49	
acetone	19,09	22,11	2,03	4,79	30,24	21,72	
ethanol	45,93	22,96	1,23	2,72	11,11	16,05	(500 µg/mL)
	45,96	22,98	1,24	2,48	11,18	16,15	(200 µg/mL)

Data expressed in percentage of total fatty acids (%).

TABLE 11. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (sugg sted proc dure)

STEP	CONDITIONS
Grinding (if particles > 5mm)	4°C
Lipid extraction	sample-acetone ratio of 1:6 (w/v) 2h (including swirling 20 min) 4°C
Filtration	organic solvent resistant filter under reduced pressure
Washing	sample-acetone ratio of 1:2 (w/v) pure and cold acetone
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure
Oil-water separation	4°C
Lipid extraction	sample-ethanol ratio of 1:2 (w/v) pure ethanol 30 min 4°C
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure

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Although the present invention has been described herein above by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A method for extracting lipids from an aquatic animal tissue comprising the steps of:

a) suspending said animal aquatic tissue in an organic solvent;

b) extracting lipids by successive organic solvent treatment; and

c) collecting said lipids in a first fraction and an organic insoluble fraction.

2. The method of claim 1, wherein said organic solvent of a) is acetone.

3. The method of claim 1 or 2, wherein said organic solvent of b) is selected from at least one of acetone and alcohol.

4. The method of claim 1, 2 or 3, wherein said organic insoluble fraction comprises a dry residue fraction which is enriched in protein.

5. The method of claim 1, 2, 3 or 4, wherein said aquatic animal tissue is at least one tissue selected from the group consisting of krill tissue, *Calanus* tissue and fish tissue.

6. A lipid extract obtained by the method of claim 2, 3, 4 or 5.

7. A protein rich fraction obtained by the method of claim 4 or 5.

8. A lipid extract having the properties in accordance with the present invention.

CONFIDENTIEL

Injection Date : 98-03-24 20:09:39
Sample Name : 7
Acq. Operator : Chantal Beaudoin

Seq. Line : -
Vial : 1
Inj : 1
Inj Volume : Manually

Method : C:\HPCHEM\1\METHODS\ALAIN2.M
Last changed : 98-03-24 19:56:07 by Chantal Beaudoin
(modified after loading)

Méthode corrigée lors de l'installation de la nouvelle colonne 12 septembre 1997. Température du four 170 degré C et purge flow = 150 ml/min. Flux dans la colonne : 4,0 ml/min. Augmentation de la température a 175 degré C et le purge flow est descendu a 140 ml/min, le 13 mars 1998.

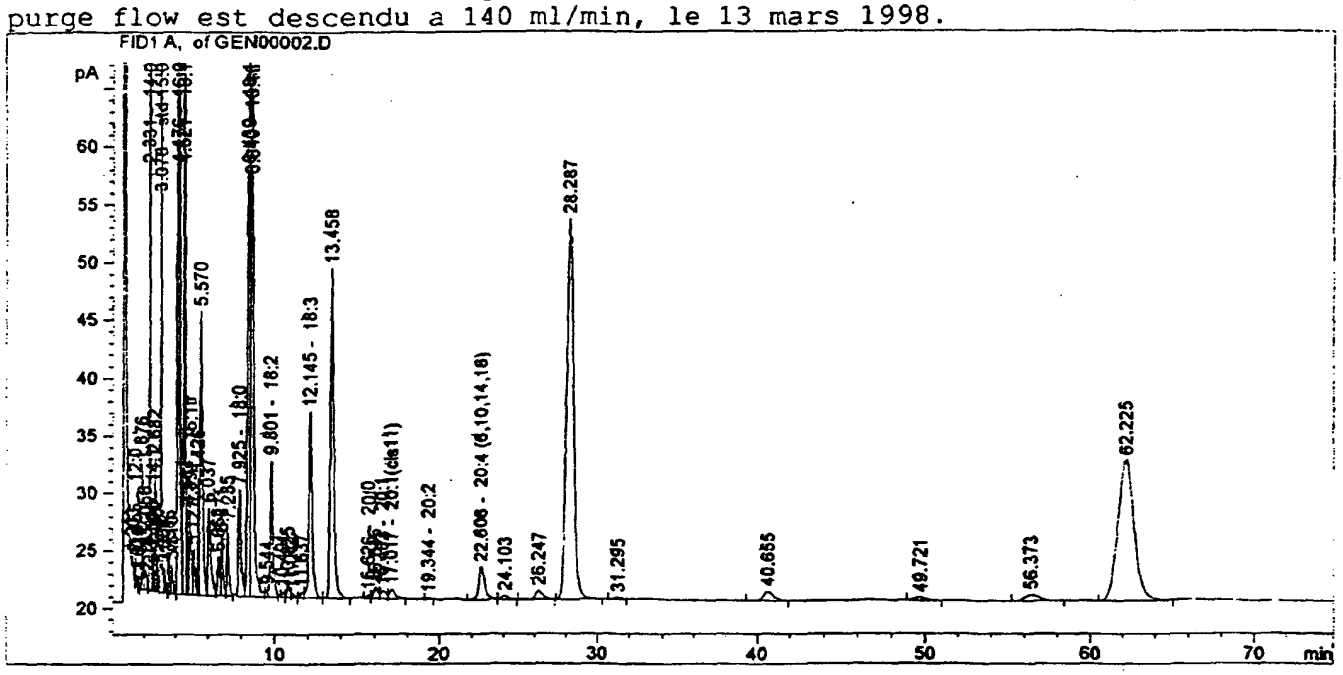

Figure 1: Gas-liquid chromatography of fatty acids from dry krill (chloroform-methanol).

CONFIDENTIEL

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Acq. Operator : Chantal Beaudoin Inj : 1
Inj Volume : Manually

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Last changed : 98-03-25 18:55:58 by Chantal Beaudoin
(modified after loading)

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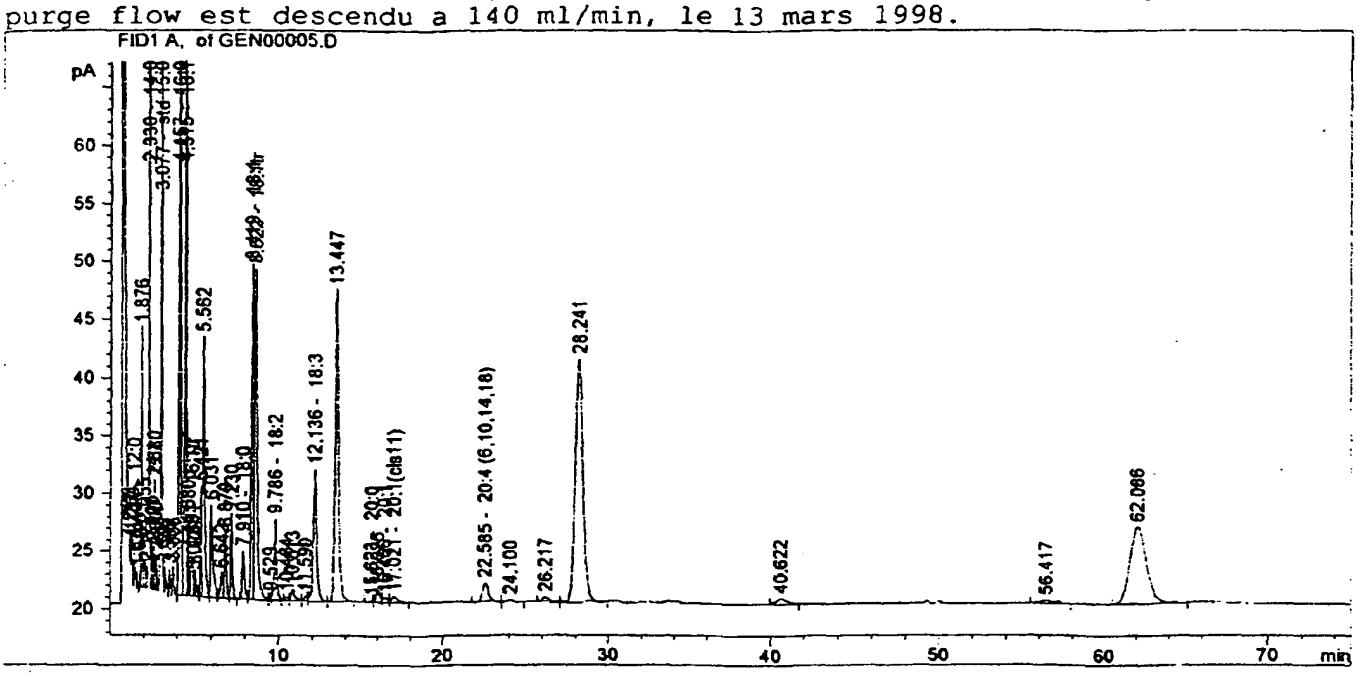

Figure 2: Gas-liquid chromatography of fatty acids from dry krill (acetone).

CONFIDENTIEL

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Acq. Operator : Chantal Beaudoin

Seq. Line : -
Vial : 1
Inj : 1
Inj Volume : Manually

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(modified after loading)

Méthode corrigée lors de l'installation de la nouvelle colonne 12 septembre 1997. Température du four 170 degré C et purge flow = 150 ml/min. Flux dans la colonne : 4,0 ml/min. Augmentation de la température a 175 degré C et le purge flow est descendu a 140 ml/min, le 13 mars 1998.

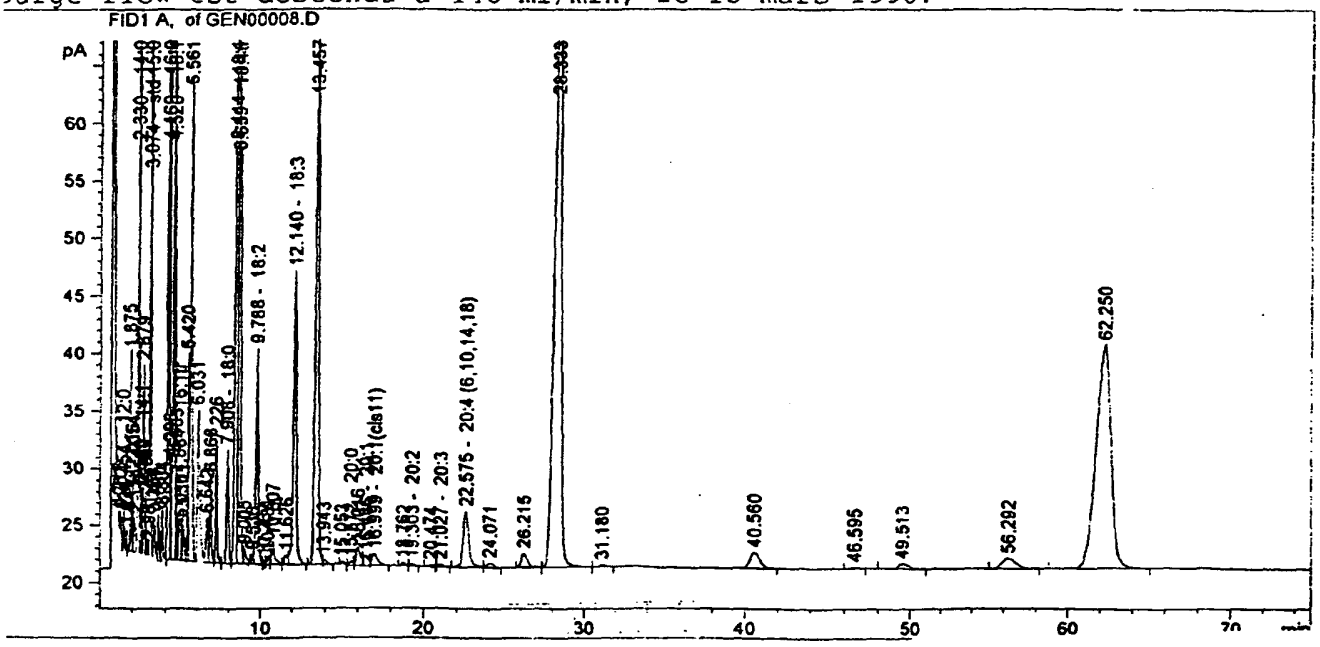

Figure 3: Gas-liquid chromatography of fatty acids from frozen krill (acetone).

CONFIDENTIEL

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Sample Name : 28
Acq. Operator : Chantal Beaudoin

Seq. Line : -
Vial : 1
Inj : 1
Inj Volume : Manually

Method : C:\HPCHEM\1\METHODS\ALAIN2.M
Last changed : 98-04-02 17:28:39 by Chantal Beaudoin
(modified after loading)

Méthode corrigée lors de l'installation de la nouvelle colonne 12 septembre 1997. Température du four 170 degré C et purge flow = 150 ml/min. Flux dans la colonne : 4,0 ml/min. Augmentation de la température a 175 degré C et le purge flow est descendu a 140 ml/min, le 13 mars 1998.

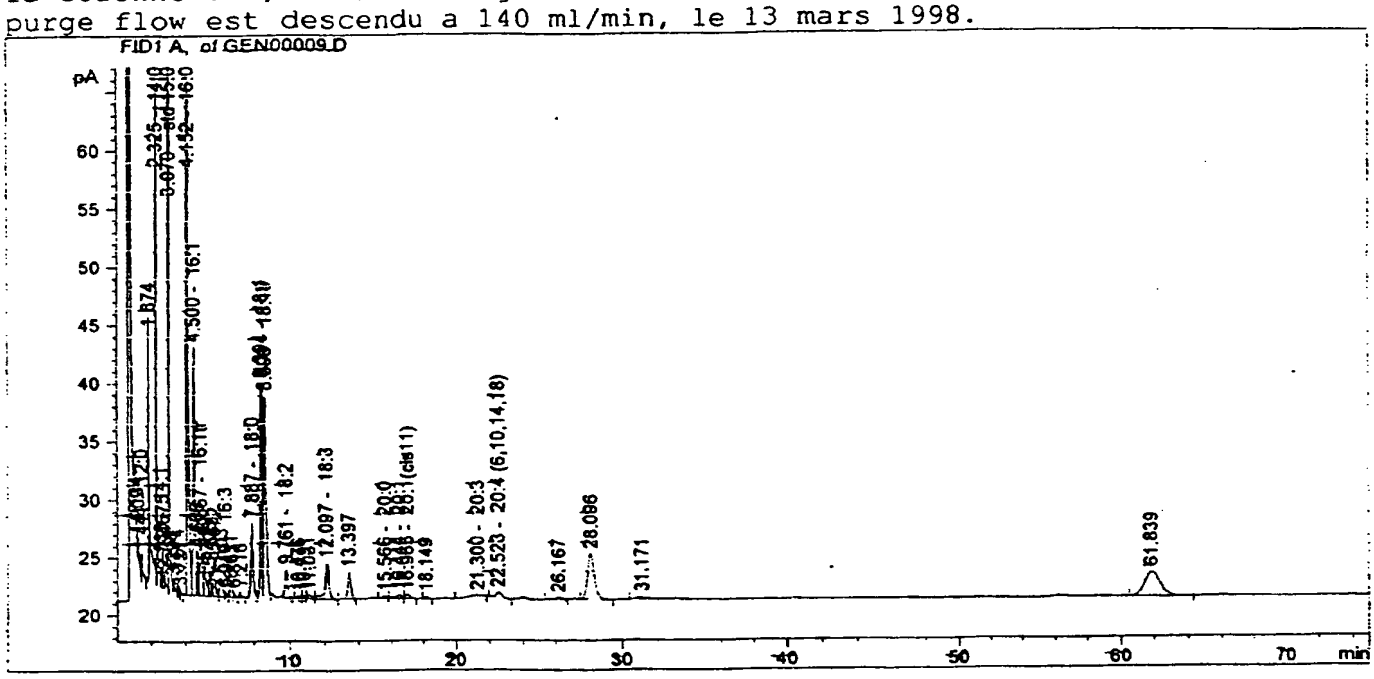


Figure 4: Gas-liquid chromatography of fatty acids from frozen krill (ethanol).

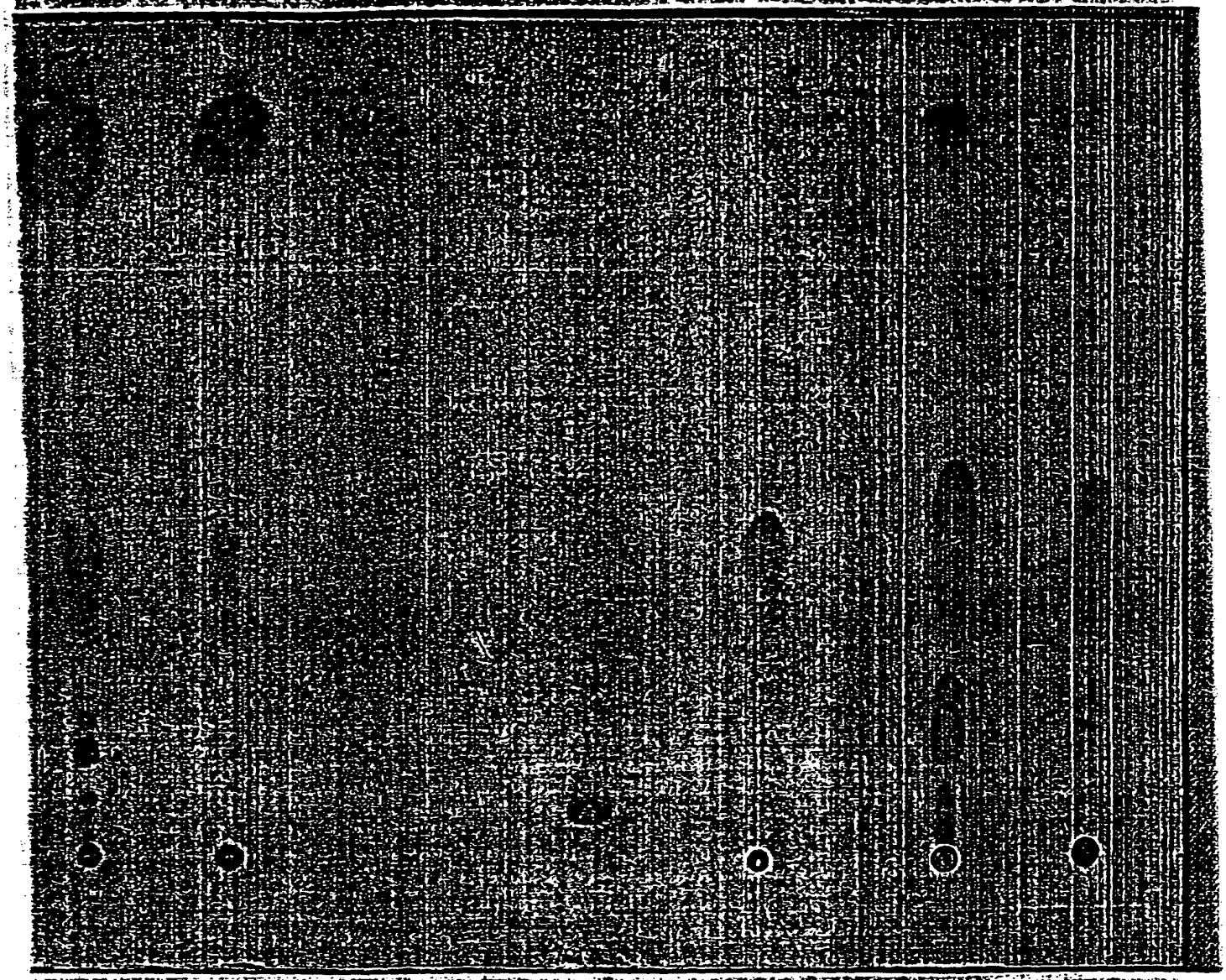


Figure 5: Thin-layer chromatography of neutral lipids of *Calanus* sp. (acetone), *Calanus* sp. (ethanol), sample of other interest, cholesterol 20mg/mL, egg (acetone), *M. norvegiça* (acetone) and *M. norvegica* (ethanol). Hexane-ethyl ether-acetic acid (90:10:1, v/v).

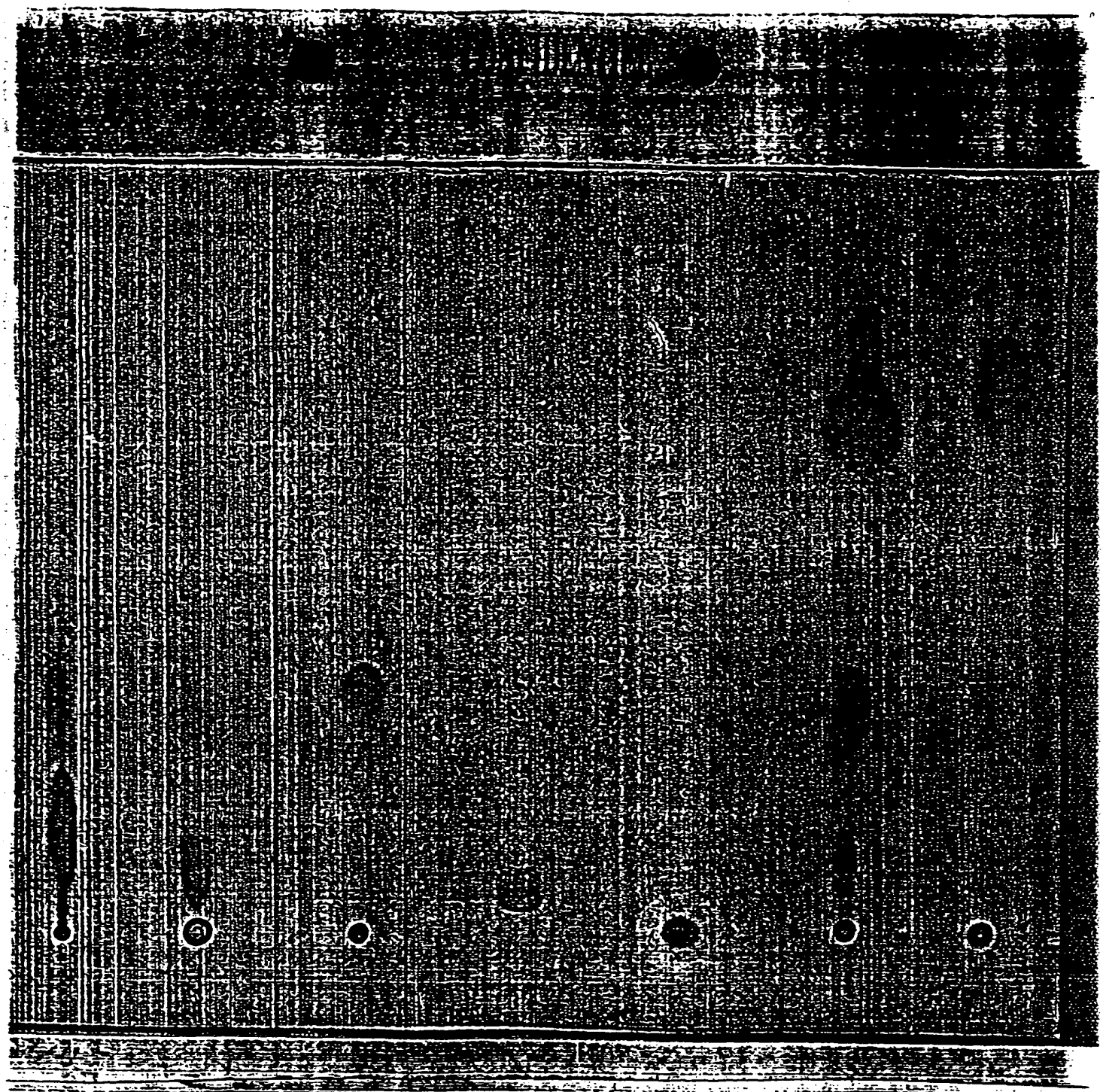


Figure 6: Thin-layer chromatography of neutral lipids of *E. pacifica* (acetone), *E. Pacifica* (ethanol), egg (acetone), cholesterol 20 mg/mL, sample of other interest, *Calanus* sp. (acetone) and *Calanus* sp. (ethanol). Hexane-ethyl ether-acetic acid (90:10:1, v/v).

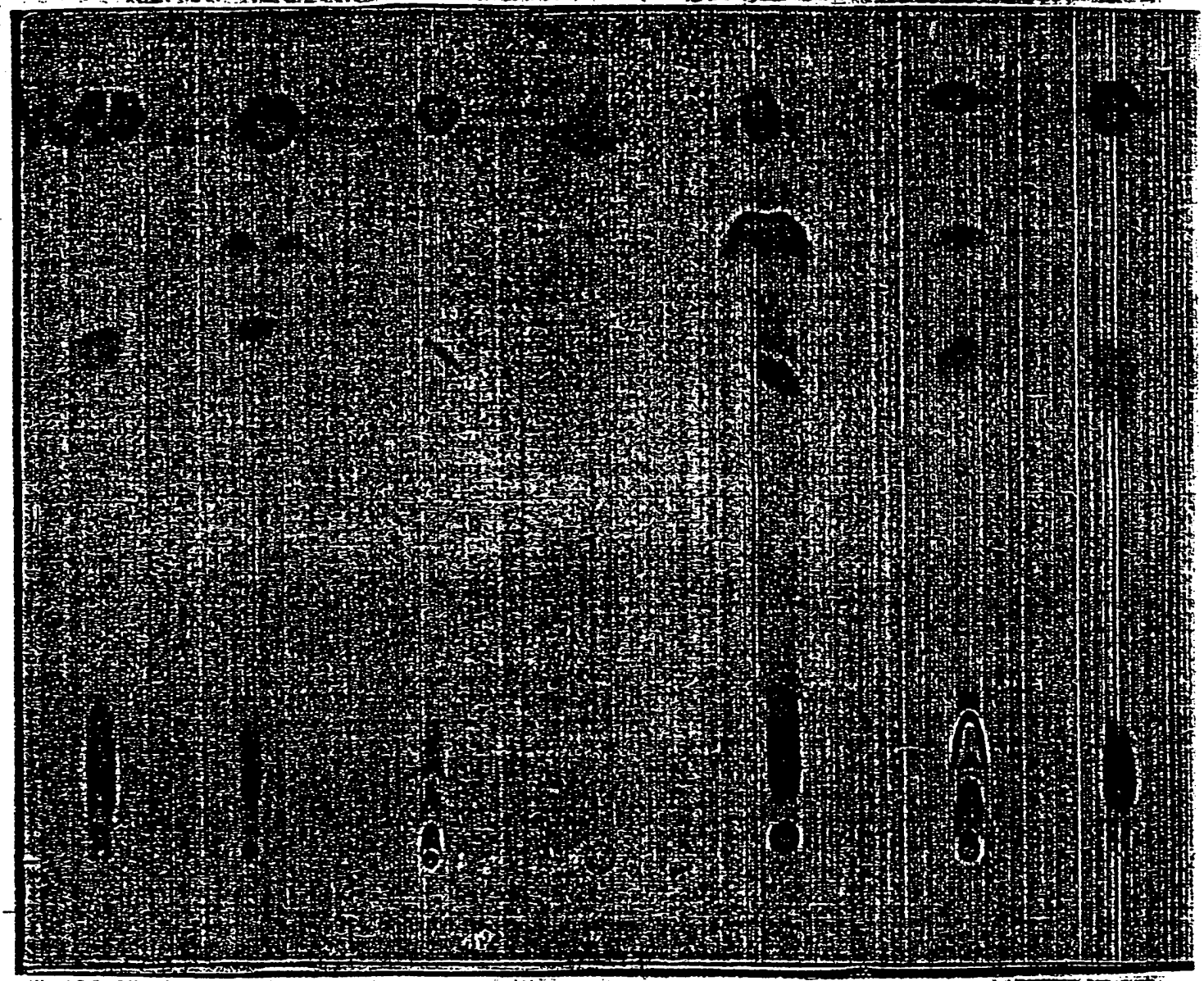


Figure 7: Thin-layer chromatography of phospholipids of *Calanus* sp. (acetone), *Calanus* sp. (ethanol), cholesterol 20 mg/mL, *M. norvegica* (acetone), *M. norvegica* (ethanol) and egg (acetone). Chloroform-methanol-water (80:25:2, v/v).

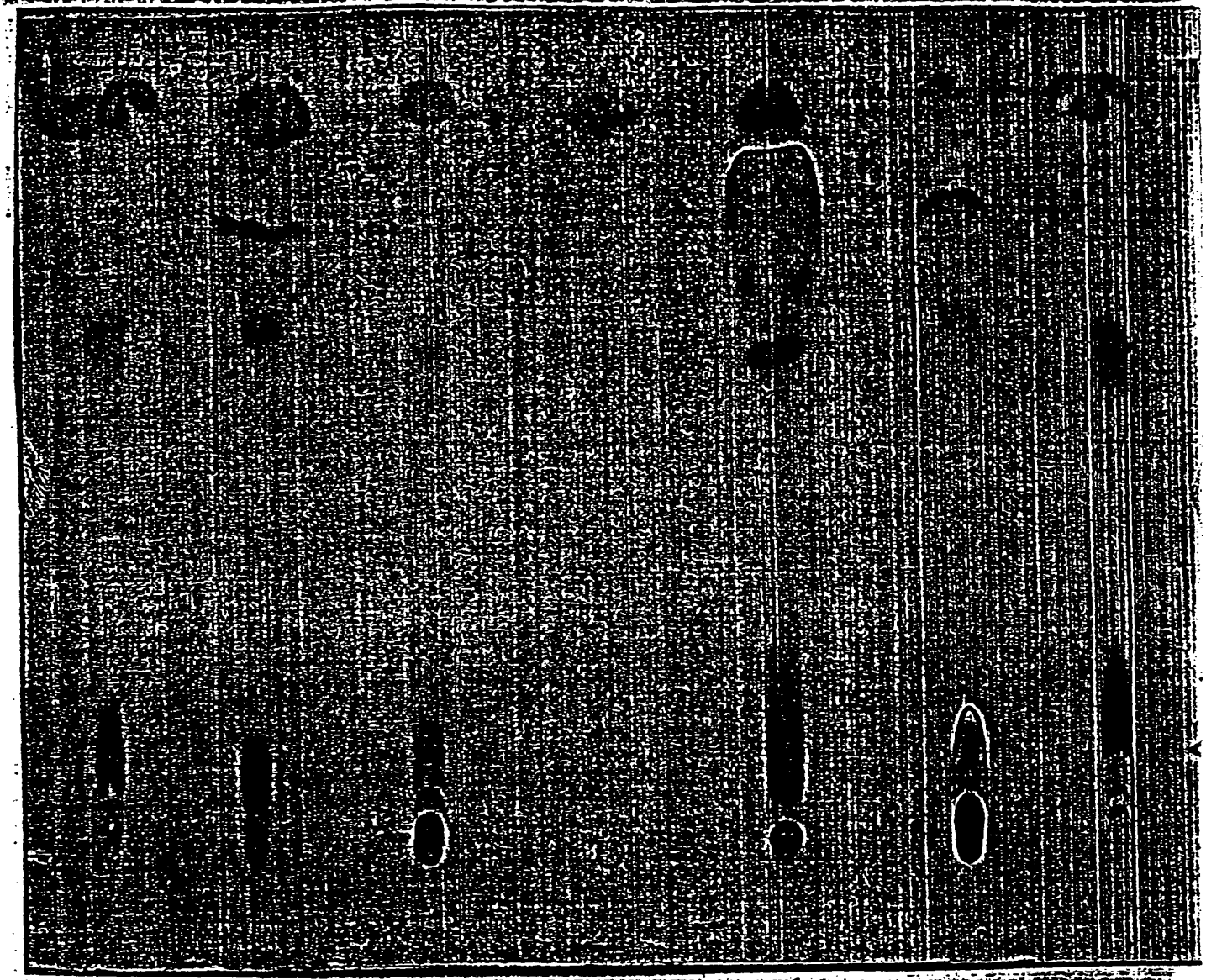


Figure 8: Thin-layer chromatography of phospholipids of *Calanus* sp. (acetone), *Calanus* sp. (ethanol), cholesterol 20 mg/mL, *E. pacifica* (acetone), *E. pacifica* (ethanol) and egg (acetone). Chloroform-metahnol-water (80:25:2), v/v).

FIGURE 9. INFLUENCE OF INCUBATION TIME IN ACETONE ON LIPID EXTRACTION (E. pacifica).

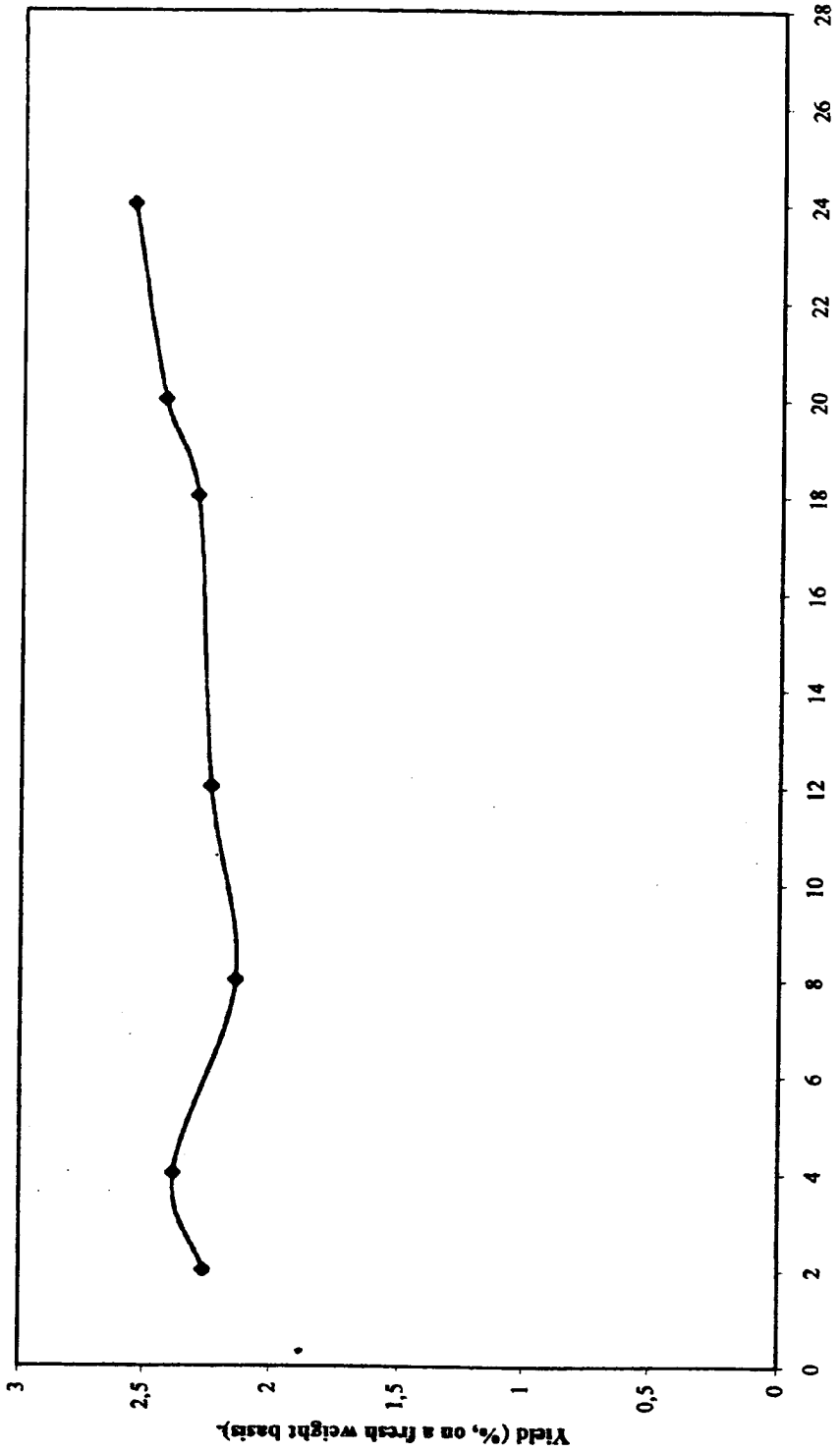
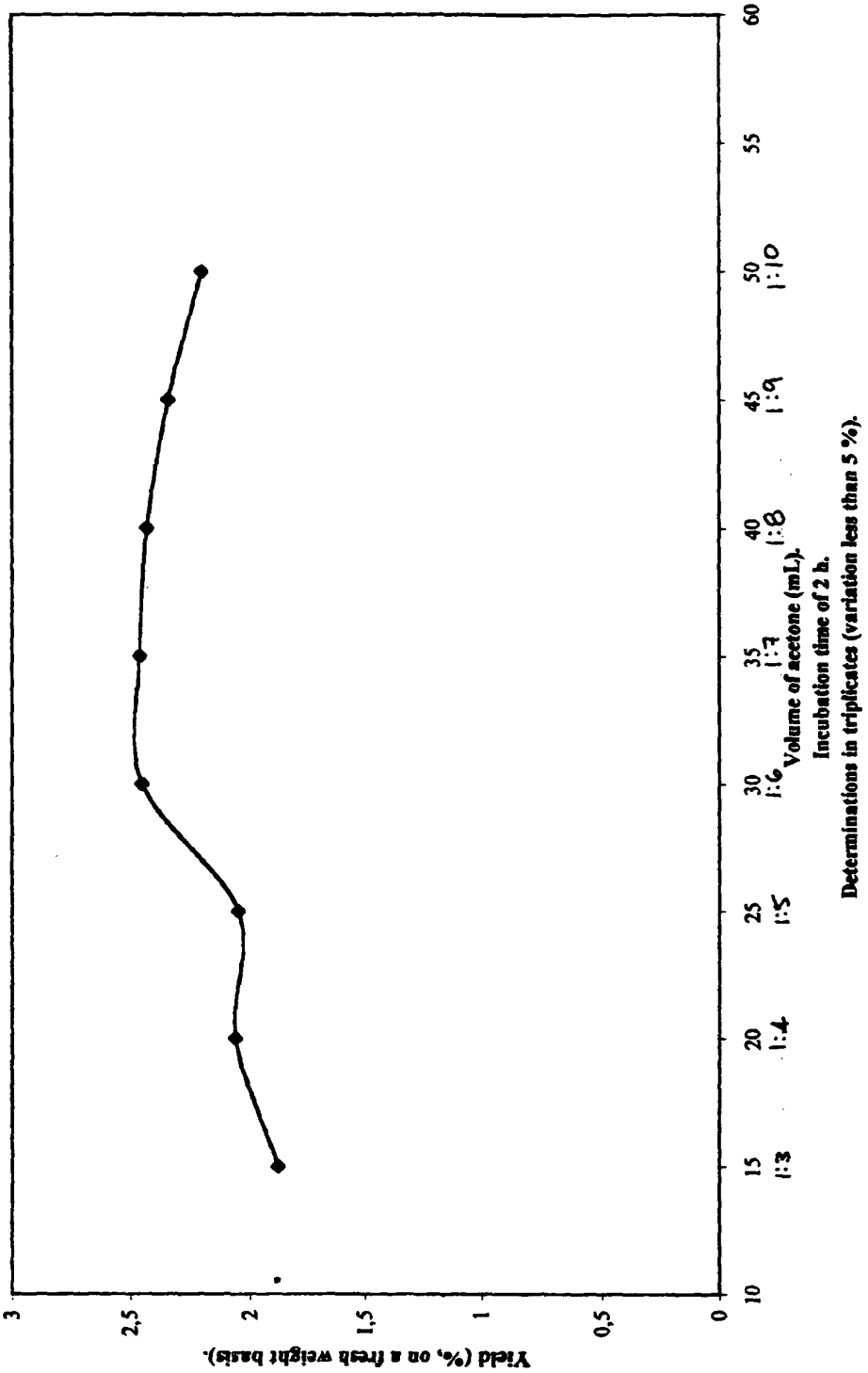


FIGURE 10. INFLUENCE OF THE VOLUME OF ACETONE ON LIPID EXTRACTION (E. pacifica).



Incubation time of 2 h.

Determinations in triplicates (variation less than 5 %).

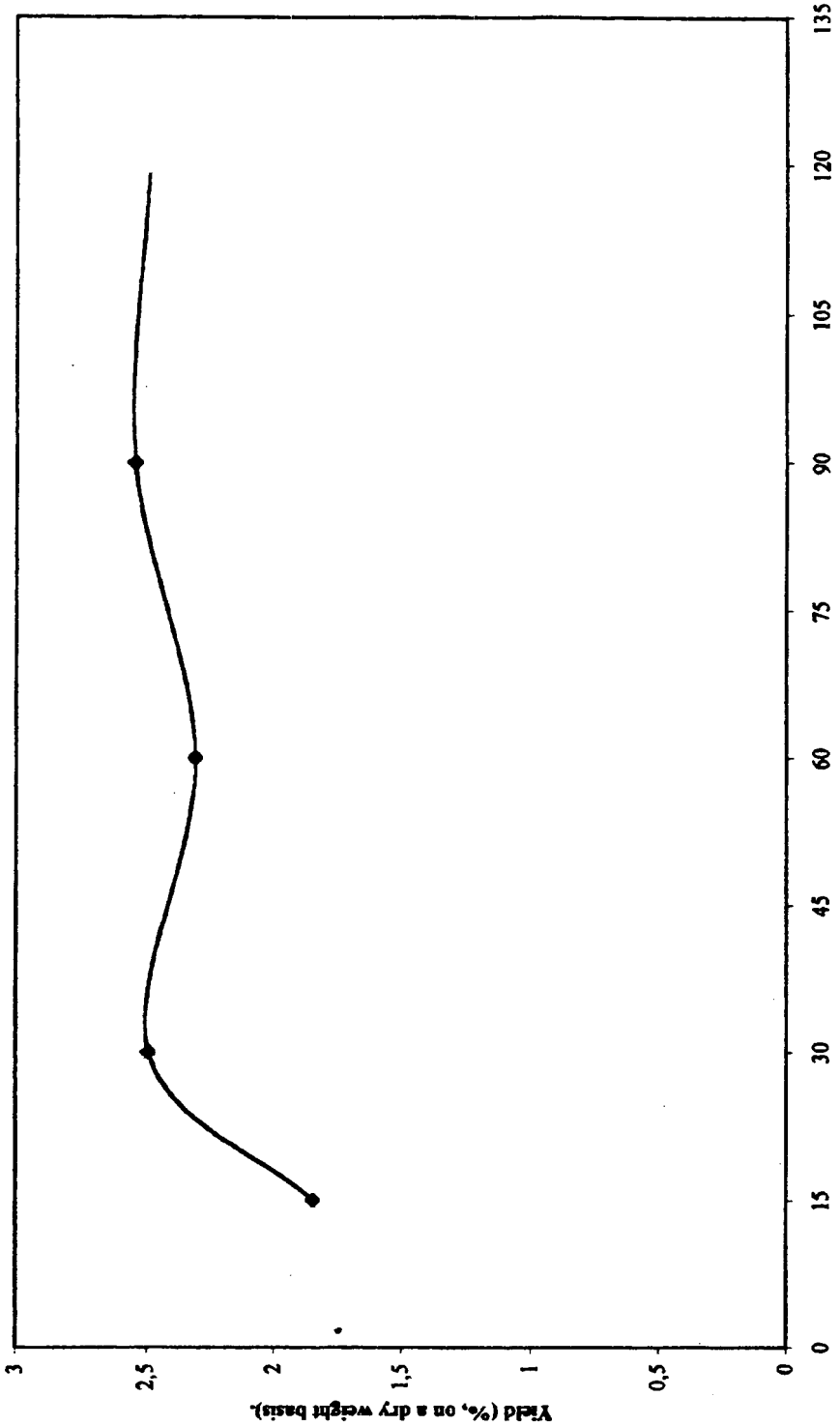


FIGURE 11. INFLUENCE OF INCUBATION TIME IN ETHANOL ON LIPID EXTRACTION (T. raschii).

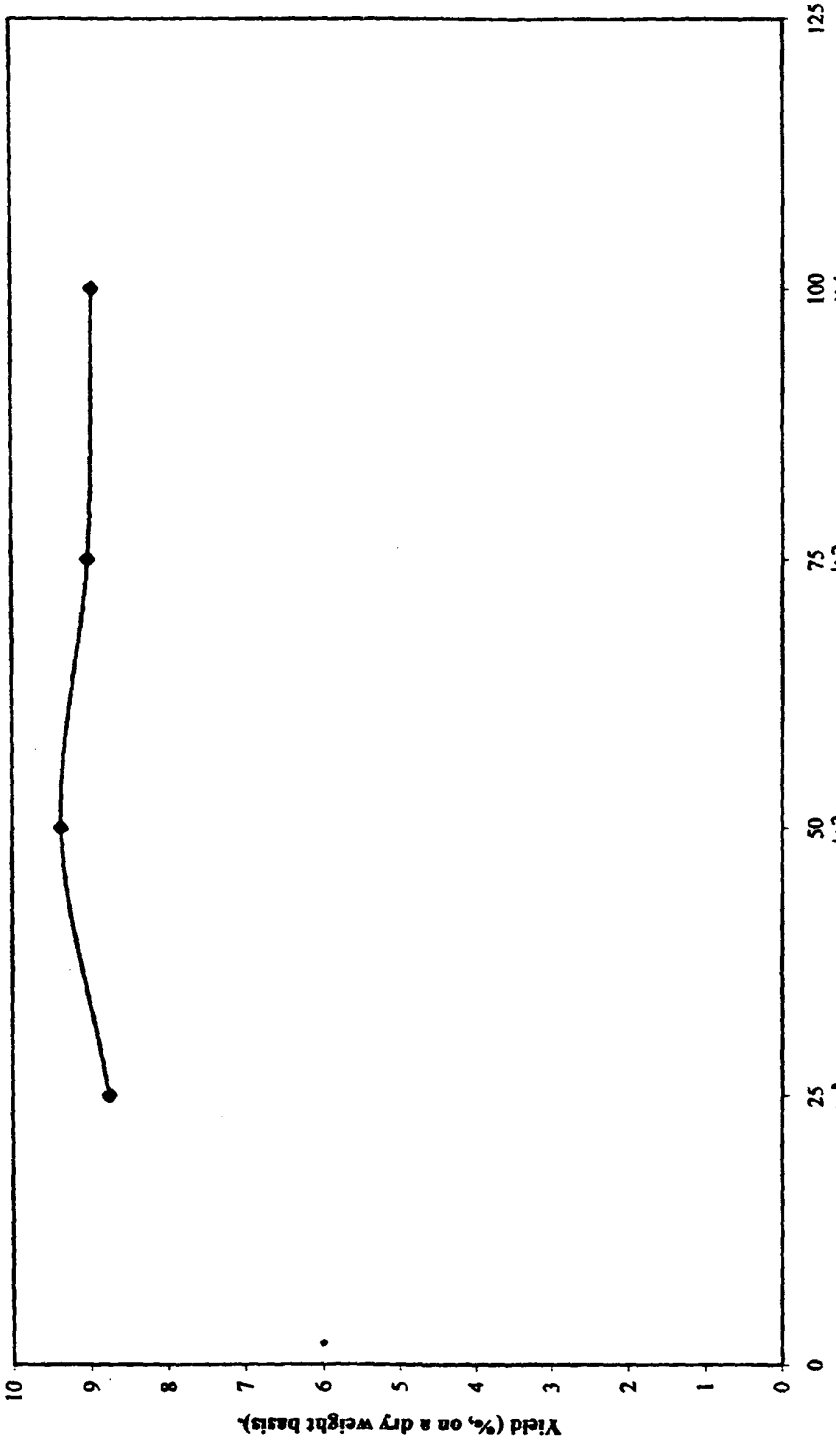


FIGURE 12. INFLUENCE OF THE VOLUME OF ETHANOL ON LIPID EXTRACTION (E. pacifica).